



5                   A NOVEL METHOD FOR USING PHAGOCYTOTIC PARTICLES  
                  AND ATP RECEPTORS TO DELIVER ANTIGENS TO MHC  
                  CLASS I RECEPTORS TO INDUCE IMMUNITY AGAINST  
                  MICROBIAL PATHOGENS OR TUMORS OR TO SUPPRESS IMMUNITY

11 Preliminary work for the invention disclosed was herein  
made in the course of work under NIH Grant No. AI 20516.  
Accordingly, the U.S. Government has certain rights in  
this invention.

17 Throughout this application, various references are  
referred to within parentheses. Disclosures of these  
publications in their entireties are hereby incorporated  
by reference into this application to more fully describe  
the state of the art to which this invention pertains.  
Full bibliographic citation for these references may be  
found at the end of this application, preceding the  
claims.

23                   **BACKGROUND OF THE INVENTION**

29 To initiate an adaptive immune response, antigen  
presenting cells (APCs) must process "foreign" proteins  
into peptides. These peptides associate with MHC  
proteins which transport these peptides to the APCs'  
plasma membrane where they are recognized in the context  
of MHC proteins by helper and cytotoxic T-cell  
precursors. Helper T-lymphocyte precursors recognize  
peptide in association with Class II MHC proteins while  
cytotoxic T-lymphocyte (CTL) precursors recognize peptide

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Scavenger receptors present on the plasma membranes of APCs promote the phagocytosis of apoptotic cells. Following their ingestion, apoptotic cells are sequestered and lysed within phagolysosomes of these APCs. This releases both ATP and various peptides into

the vacuole of the the APCs' phago-lysosome. It is  
hypothesized that the ATP released from apoptotic cells  
into phagolysosomes of APCs opens P2X<sub>7</sub> receptors. This  
provides a pathway by which potentially immunogenic  
5 peptides from "foreign" apoptotic cells, and potentially  
"toleragenic" peptides from self apoptotic cells, enter  
the cytoplasm of APCs. These peptides then can be  
carried by TAP proteins into the endoplasmic reticulum  
where they associate with Class I MHC proteins. APCs and  
especially immature dendritic cells (1), recycle Class II  
11 MHC molecules from their phago-lysosomes to the plasma  
membrane. Thus peptide antigens released into phago-  
lysosomes are efficiently presented in association with  
Class II MHC proteins.

Antigen presenting cells (APCs) whose Class I and Class  
17 II MHC molecules contain antigen peptides elicit  
cytotoxic and helper T-lymphocytes. In some instances,  
these cytotoxic and helper lymphocytes cause tumor  
regression. Devised herein is a novel method for  
delivery of immunogenic peptides to macrophages and  
dendritic cells for presentation by Class I and Class II  
23 MHC proteins. The method uses as a delivery vehicle IgG-  
opsonized resealed red blood cell ghosts (rRBCg)  
containing immunogenic peptides for delivery to Class II  
MHC proteins, and IgG-opsonized-rRBCg containing  
immunogenic peptides and ATP for delivery to Class I MHC  
29 proteins. In the latter instance, the method makes use  
of ATP<sup>4-</sup>-activated receptors (which may be P2X<sub>7</sub>, or other  
ATP receptors) present in phagolysosomal vesicles to  
deliver immunogenic peptides to the cytoplasmic matrix of

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Human red blood cell ghosts or other particles that can be filled with antigens (e.g., liposomes) and coated with ligands (IgG, oxidized lipids, sugars, polyanions), for receptors on antigen presenting cells (e.g., dendritic cells, Langerhans cells, monocytes, macrophages), are used as vehicles to encapsulate antigens (e.g., peptides, carbohydrates lipids, glycoproteins, glycolipids, lipoproteins), and adenosine triphosphate (ATP) or other ligands for ATP receptors (e.g. P2X7 and other ATP receptors)]. The antigens may be an antigen derived from, and/or induce immune responses that affect microbial pathogens, tumor cells, and/or immunoregulatory pathways. Ligands on the particle will promote ingestion of the particle by antigen presenting cells. Enzymes released into particle-containing phagosomes of antigen presenting cells will lyse the particle, releasing ATP and/or other substances that activate ATP receptors (such as P2X7 receptors, but not limited to these receptors) into these phagosomes. Activation of the receptors will create "pores" in the phagosomes' membranes through which antigens (e.g., antigenic peptides, carbohydrates, lipids) can enter the cytoplasm for processing and presentation to T-cells in association with conventional Class I MHC molecules, or other antigen presenting receptors.

The invention disclosed herein is useful as a vaccine, as a method for delivery of antigens to the cytoplasmic matrix of antigen presenting cells to induce immunity, to

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## SUMMARY OF THE INVENTION

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5 This invention provides a method of delivering an antigen to an Class I MHC receptor to induce immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) 11 incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of 17 the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); and d) administering the antigen presenting cells (APCs) of step (c) to a subject having the disease so as induce Class I MHC presentation and 23 elicit cytotoxic T-lymphocytes against the antigen, thereby inducing immunity against the antigen.

29 This invention provides a method of delivering an antigen to an Class I MHC receptor to induce immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of

step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); d) incubating the Ag-APCs of step (c) with lymphocytes previously removed from the subject having the disease; and e) administering the incubated lymphocytes of step (d) to the subject so to induce immunity against the antigen in the subject.

This invention provides a method of delivering an antigen to an Class II MHC receptor to induce immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to



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5 facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class II MHC receptor and is expressed on the surface of the APCs (Ag-APCs); and d) administering the antigen presenting cells (APCs) of step (c) to a subject having the disease so as to induce Class II MHC presentation and elicit helper T-lymphocytes against the antigen, thereby inducing immunity against the antigen.

11 This invention provides a method of delivering an antigen to an Class II MHC receptor to induce immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); d) incubating the Ag-APCs of step (c) with lymphocytes previously removed from the subject having the disease; and e) administering the incubated lymphocytes of step (d) to the subject so as to induce Class II MHC presentation and elicit helper T-

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This invention provides a method of delivering an antigen to an Class I MHC receptor to suppress immunity against the antigen in a subject having a disease which

comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); d) incubating the Ag-APCs of step (c) with lymphocytes previously removed from the subject having the disease; and e) administering the incubated lymphocytes of step (d) to the subject so as to induce Class I MHC presentation and elicit suppressor T-lymphocytes so to suppress immunity against the antigen in the subject.

This invention provides a method of delivering an antigen to an Class I MHC receptor to suppress immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-

filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); and d) administering the antigen presenting cells (APCs) of step (c) to a subject having the disease so as induce Class I MHC presentation and elicit suppressor T-lymphocytes so to suppress immunity against the antigen in the subject.

This invention provides a method of delivering an antigen to an Class II MHC receptor to suppress immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class II MHC receptor and is expressed on

the surface of the APCs (Ag-APCs); and d) administering the antigen presenting cells (APCs) of step (c) to a subject having the disease so as induce Class II MHC presentation and elicit suppressor T-lymphocytes so to suppress immunity against the antigen in the subject.

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**BRIEF DESCRIPTION OF THE FIGURES**

5                    **Figure 1: Mouse Fetal Microglial Cells.**    Mouse fetal  
                         microglial cells were incubated with IgG  
                         opsonized red blood cell resealed ghosts  
                         (containing ATP and lucifer yellow). At the  
                         indicated times, the cells were observed under  
                         fluorescence microscopy. At 60 mins and 4  
                         hours, the microglial cells ingested the  
                         particles and the dye is still contained within  
11                    red blood cell ghosts. However, within 24 hrs,  
                         the dye has left the phagolysosome and now  
                         appears throughout the cytoplasm of the cells.  
                         (LY=lucifer yellow)

17                    **Figure 2: Human Monocyte Derived Dendritic Cells.** At 24  
                         hours, the dendritic cells that have ingested  
                         the IgG opsonized red blood cell resealed  
                         ghosts (containing ATP and lucifer yellow) now  
                         express the dye throughout the cytoplasm of the  
                         cell.

23                    **Figure 3: B6 Bone Marrow Derived Dendritic Cells 3 hr Fl-**  
                         **Ova-E(IgG).**        Mouse Bone marrow derived  
                         dendritic cells were incubated for about 4  
                         hours with IgG opsonized red blood cell  
                         resealed ghosts (containing ATP and  
                         fluorochrome conjugated ovalbumin peptide [fl-  
29                    ova]). In many of the cells, the fluorochrome  
                         dye is observed to be localized throughout the  
                         cytoplasm rather than in phagolysosomes.



**DETAILED DESCRIPTION OF THE INVENTION**

5 This invention provides a method of delivering an antigen to an Class I MHC receptor to induce immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) 11 incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into 17 cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); and d) administering the antigen presenting cells (APCs) of step (c) to a subject having the disease so as induce Class I MHC presentation and 23 elicit cytotoxic T-lymphocytes against the antigen, thereby inducing immunity against the antigen.

29 In an embodiment of the above-described method of delivering an antigen to an Class I MHC receptor to induce immunity against the antigen in a subject having a disease, the particle is a type O red blood cell ghost. In another embodiment of the method the particle is a liposome. In an embodiment the ligand is selected from



the group consisting of an immunoglobulin (IgG), complement component C3b, complement component C3bi, maleic anhydride, an oxidized lipid, a sugar, and a polyanion. In a further embodiment the antigen presenting cell is selected from the group consisting of a dendritic cell, a Langerhans cell, a monocyte, a mononuclear phagocyte, a macrophage, a Kupfer cell, a microglial cell, an osteoclast, and a bone marrow-derived leukocyte. In an embodiment the antigen is a purified antigen. In preferred embodiments the antigen is a cancer cell antigen, a bacterial antigen or a viral antigen. In an embodiment the antigen is a crude cell extract. In additional preferred embodiments the antigen is a cancer cell antigen, a bacterial antigen or a viral antigen. In an embodiment the antigen is selected from the group consisting of a peptide, a carbohydrate, a lipid, a glycoprotein, a glycolipid and a lipoprotein. In another embodiment of the above-described methods, the method further comprises delivering at least one stimulatory cytokine with the antigen to the cytoplasmic matrix of an antigen presenting cell which comprises in step (a) filling the particle with the stimulatory cytokine. In an embodiment the cytokine is IL-12, G-CSF, IL-4, GM-CSF or interferon gamma. In preferred embodiments the immunity induced is against a bacterial or viral antigen. In still further preferred embodiments the immunity induced is against a cancerous tumor. In preferred embodiments the disease is a bacterial infection or a viral infection. In additional preferred embodiments the disease is cancer.

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5 This invention provides a method of delivering an antigen to an Class I MHC receptor to induce immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); d) incubating the Ag-APCs of step (c) with lymphocytes previously removed from the subject having the disease; and e) administering the incubated lymphocytes of step (d) to the subject so to induce immunity against the antigen in the subject.

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23 In an embodiment of the above-described method of delivering an antigen to an Class I MHC receptor to induce immunity against the antigen in a subject having a disease, the particle is a type O red blood cell ghost. In an embodiment the particle is a liposome. In another

29 embodiment the ligand is selected from the group consisting of an immunoglobulin (IgG), complement component C3b, complement component C3bi, maleic

anhydride, an oxidized lipid, a sugar, and a polyanion. In a further embodiment the antigen presenting cell is selected from the group consisting of a dendritic cell, a Langerhans cell, a monocyte, a mononuclear phagocyte, a macrophage, a Kupfer cell, a microglial cell, an osteoclast, and a bone marrow-derived leukocyte. In an embodiment the antigen is a purified antigen. In a preferred embodiment the purified antigen is a cancer cell antigen, a bacterial antigen or a viral antigen. In an embodiment the antigen is a crude cell extract. In another preferred embodiment the crude cell extract antigen is a cancer cell antigen, a bacterial antigen or a viral antigen. In an embodiment the antigen is selected from the group consisting of a peptide, a carbohydrate, a lipid, a glycoprotein, a glycolipid and a lipoprotein. In another embodiment of the above-described method the method further comprises delivering at least one stimulatory cytokine with the antigen to the cytoplasmic matrix of an antigen presenting cell which comprises in step (a) filling the particle with the stimulatory cytokine. In an embodiment the cytokine is IL-12, G-CSF, IL-4, GM-CSF or interferon gamma. In a preferred embodiment the immunity induced is against a bacterial or viral antigen. In another preferred embodiment the immunity induced is against a cancerous tumor. In a further preferred embodiment the disease is a bacterial infection or a viral infection. In a still further preferred embodiment the disease is cancer.

This invention provides a method of delivering an antigen to an Class II MHC receptor to induce immunity against

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5 the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class II MHC receptor and is expressed on the surface of the APCs (Ag-APCs); and d) administering the antigen presenting cells (APCs) of step (c) to a subject having the disease so as induce Class II MHC presentation and elicit helper T-lymphocytes against the antigen, thereby inducing immunity against the antigen.

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23 In an embodiment of the above-described method of delivering an antigen to an Class II MHC receptor to induce immunity against the antigen in a subject having a disease the particle is a type O red blood cell ghost. In an embodiment the particle is a liposome. In another embodiment the ligand is selected from the group consisting of an immunoglobulin (IgG), complement component C3b, complement component C3bi, maleic anhydride, an oxidized lipid, a sugar, and a polyanion.

29 In a further embodiment the antigen presenting cell is

selected from the group consisting of a dendritic cell, a Langerhans cell, a monocyte, a mononuclear phagocyte, a macrophage, a Kupfer cell, a microglial cell, an osteoclast, and a bone marrow-derived leukocyte. In an embodiment the antigen is a purified antigen. In a preferred embodiment the purified antigen is a cancer cell antigen, a bacterial antigen or a viral antigen. In an embodiment the antigen is a crude cell extract. In an embodiment the crude cell extract antigen is a cancer cell antigen, a bacterial antigen or a viral antigen. In an embodiment the antigen is selected from the group consisting of a peptide, a carbohydrate, a lipid, a glycoprotein, a glycolipid and a lipoprotein. In an embodiment of the above-described methods, the method further comprising delivering at least one stimulatory cytokine with the antigen to the cytoplasmic matrix of an antigen presenting cell which comprises in step (a) filling the particle with the stimulatory cytokine. In an embodiment the cytokine is IL-12, G-CSF, IL-4, GM-CSF or interferon gamma. In a preferred embodiment the immunity induced is against a bacterial or viral antigen. In another preferred embodiment the immunity induced is against a cancerous tumor. In other preferred embodiments the disease is a bacterial infection or a viral infection. In further preferred embodiment the disease is cancer.

This invention provides a method of delivering an antigen to an Class II MHC receptor to induce immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP

resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); d) incubating the Ag-APCs of step (c) with lymphocytes previously removed from the subject having the disease; and e) administering the incubated lymphocytes of step (d) to the subject so as to induce Class II MHC presentation and elicit helper T-lymphocytes so to induce immunity against the antigen in the subject.

In an embodiment of the above-described method of delivering an antigen to an Class II MHC receptor to induce immunity against the antigen in a subject having a disease the particle is a type O red blood cell ghost. In an embodiment the particle is a liposome. In another embodiment the ligand is selected from the group consisting of an immunoglobulin (IgG), complement component C3b, complement component C3bi, maleic anhydride, an oxidized lipid, a sugar, and a polyanion. In a further embodiment the antigen presenting cell is

selected from the group consisting of a dendritic cell, a Langerhans cell, a monocyte, a mononuclear phagocyte, a macrophage, a Kupfer cell, a microglial cell, an osteoclast, and a bone marrow-derived leukocyte. In an embodiment the antigen is a purified antigen. In a preferred embodiment the purified antigen is a cancer cell antigen, a bacterial antigen or a viral antigen. In another embodiment the antigen is a crude cell extract. In a preferred embodiment the crude cell extract antigen is antigen is a cancer cell antigen, a bacterial antigen or a viral antigen. In an embodiment the antigen is selected from the group consisting of a peptide, a carbohydrate, a lipid, a glycoprotein, a glycolipid and a lipoprotein. In another embodiment of the above-described methods, the method further comprises delivering at least one stimulatory cytokine with the antigen to the cytoplasmic matrix of an antigen presenting cell which comprises in step (a) filling the particle with the stimulatory cytokine. In an embodiment the cytokine is IL-12, G-CSF, IL-4, GM-CSF or interferon gamma. In a preferred embodiment the immunity induced is against a bacterial or viral antigen. In another preferred embodiment the immunity induced is against a cancerous tumor. In further preferred embodiment the disease is a bacterial infection or a viral infection. In another preferred embodiment the disease is cancer.

This invention provides a method of delivering an antigen to an Class II MHC receptor to suppress immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP

resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class II MHC receptor and is expressed on the surface of the APCs (Ag-APCs); d) incubating the Ag-APCs of step (c) with lymphocytes previously removed from the subject having the disease; and e) administering the incubated lymphocytes of step (d) to the subject so as to induce Class II MHC presentation and elicit suppressor T-lymphocytes so to suppress immunity against the antigen in the subject.

In an embodiment of the above-described method of delivering an antigen to an Class II MHC receptor to suppress immunity against the antigen in a subject having a disease the particle is a type O red blood cell ghost. In an embodiment the particle is a liposome. In another embodiment the ligand is selected from the group consisting of an immunoglobulin (IgG), complement component C3b, complement component C3bi, maleic anhydride, an oxidized lipid, a sugar, and a polyanion. In a further embodiment the antigen presenting cell is





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coated Ag/ATP-filled particles and APC phagolysosomes to  
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phagolysosomes into cytoplasm such that the antigen is  
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consisting of an immunoglobulin (IgG), complement component C3b, complement component C3bi, maleic anhydride, an oxidized lipid, a sugar, and a polyanion. In another embodiment the antigen presenting cell is selected from the group consisting of a dendritic cell, a Langerhans cell, a monocyte, a mononuclear phagocyte, a macrophage, a Kupfer cell, a microglial cell, an osteoclast, and a bone marrow-derived leukocyte. In an embodiment the antigen is a purified antigen. In a preferred embodiment of the purified antigen, the antigen is an antigen of a transplant organ. In an embodiment the transplant organ antigen is allogeneic antigen, a syngeneic antigen, or a xenogenic antigen. In another embodiment the antigen is a purified antigen the antigen is a crude cell extract. In a preferred embodiment of the crude cell extract antigen, the antigen is an antigen of a transplant organ. In an embodiment the transplant organ antigen is allogeneic antigen, a syngeneic antigen, or a xenogenic antigen. In another embodiment the antigen is selected from the group consisting of a peptide, a carbohydrate, a lipid, a glycoprotein, a glycolipid and a lipoprotein. In an embodiment of the above-described methods, the method further comprises delivering at least one stimulatory cytokine with the antigen to the cytoplasmic matrix of an antigen presenting cell which comprises in step (a) filling the particle with the stimulatory cytokine. In an embodiment the cytokine is IL-12, G-CSF, IL-4, GM-CSF or interferon gamma. In a preferred embodiment the immunity suppressed is immunity against a transplanted organ or tissue. In other preferred embodiments the immunity suppressed is immunity

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against organs of the subject. In additional preferred embodiments the disease is an autoimmune disease or rejection of a transplanted organ or tissue.

5 This invention provides a method of delivering an antigen to an Class I MHC receptor to suppress immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class I MHC receptor and is expressed on the surface of the APCs (Ag-APCs); and d) administering the antigen presenting cells (APCs) of step (c) to a subject having the disease so as induce Class I MHC presentation and elicit suppressor T-lymphocytes so to suppress immunity against the antigen in the subject.

29 In an embodiment of the above-described method of delivering an antigen to an Class I MHC receptor to suppress immunity against the antigen in a subject having a disease the particle is a type O red blood cell ghost.

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5 In another embodiment the particle is a liposome. In a further embodiment the ligand is selected from the group consisting of an immunoglobulin (IgG), complement component C3b, complement component C3bi, maleic anhydride, an oxidized lipid, a sugar, and a polyanion.

11 In yet another embodiment the antigen presenting cell is selected from the group consisting of a dendritic cell, a Langerhans cell, a monocyte, a mononuclear phagocyte, a macrophage, a Kupfer cell, a microglial cell, an osteoclast, and a bone marrow-derived leukocyte. In an embodiment the antigen is a purified antigen. In a preferred embodiment of the purified antigen the antigen is an antigen of a transplant organ. In an embodiment the transplant organ antigen is allogeneic antigen, a syngeneic antigen, or a xenogenic antigen. In another embodiment the antigen is a crude cell extract. In a preferred embodiment of the a crude cell extract the antigen is an antigen of a transplant organ. In an embodiment the transplant organ antigen is allogeneic antigen, a syngeneic antigen, or a xenogenic antigen. In another embodiment the antigen is selected from the group consisting of a peptide, a carbohydrate, a lipid, a glycoprotein, a glycolipid and a lipoprotein. In an embodiment of the above-described methods, the method further comprises delivering at least one stimulatory cytokine with the antigen to the cytoplasmic matrix of an antigen presenting cell which comprises in step (a) filling the particle with the stimulatory cytokine. In an embodiment the cytokine is IL-12, G-CSF, IL-4, GM-CSF or interferon gamma. In a preferred embodiment the immunity suppressed is immunity against a transplanted organ or

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tissue. In another preferred embodiment the immunity suppressed is immunity against organs of the subject. In a still further preferred embodiment the disease is an autoimmune disease or rejection of a transplanted organ or tissue.

11 This invention provides a method of delivering an antigen to an Class II MHC receptor to suppress immunity against the antigen in a subject having a disease which comprises: a) filling particles with the antigen and ATP resulting in an antigen- and ATP-filled particles (Ag/ATP-filled particles); b) coating the Ag/ATP-filled particles of step (a) with a ligand for an antigen presenting cell resulting in a ligand-coated Ag/ATP-filled particles; c) incubating the ligand-coated Ag/ATP-filled particles of step (b) with isolated ligand-binding antigen presenting cells (APCs) under conditions permitting the ligand-binding APCs to bind to the ligand-coated Ag/ATP-filled particles and APC phagolysosomes to ingest the ligand-coated Ag/ATP-filled particles to facilitate transfer of the ingested antigen from the phagolysosomes into cytoplasm such that the antigen is delivered to a Class II MHC receptor and is expressed on the surface of the APCs (Ag-APCs); and d) administering the antigen presenting cells (APCs) of step (c) to a subject having the disease so as induce Class II MHC presentation and elicit suppressor T-lymphocytes so to suppress immunity against the antigen in the subject.

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In the above-described method of delivering an antigen to an Class II MHC receptor to suppress immunity against the

antigen in a subject having a disease the particle is a type O red blood cell ghost. In another embodiment of the method method the particle is a liposome. In a further embodiment the ligand is selected from the group consisting of an immunoglobulin (IgG), complement component C3b, complement component C3bi, maleic anhydride, an oxidized lipid, a sugar, and a polyanion. In another embodiment the antigen presenting cell is selected from the group consisting of a dendritic cell, a Langerhans cell, a monocyte, a mononuclear phagocyte, a macrophage, a Kupfer cell, a microglial cell, an osteoclast, and a bone marrow-derived leukocyte. In yet another embodiment the antigen is a purified antigen. In a preferred embodiment of the purified antigen, the antigen is an antigen of a transplant organ. In an embodiment the transplant organ antigen is allogeneic antigen, a syngeneic antigen, or a xenogenic antigen. In another embodiment the antigen is a crude cell extract. In a preferred embodiment of the crude cell extract antigen, the antigen is an antigen of a transplant organ. In an embodiment the transplant organ antigen is allogeneic antigen, a syngeneic antigen, or a xenogenic antigen. In a further embodiment the antigen is selected from the group consisting of a peptide, a carbohydrate, a lipid, a glycoprotein, a glycolipid and a lipoprotein. In an embodiment of the above-described methods, the method further comprises delivering at least one stimulatory cytokine with the antigen to the cytoplasmic matrix of an antigen presenting cell, step (a) comprises filling the particle with the stimulatory cytokine. In additional embodiments the cytokine is IL-12, G-CSF, IL-

4, GM-CSF or interferon gamma. In a preferred embodiment  
the immunity suppressed is immunity against a  
transplanted organ or tissue. In another preferred  
embodiment the immunity suppressed is immunity against  
5 organs of the subject. In further preferred embodiments  
the disease is an autoimmune disease or rejection of a  
transplanted organ or tissue.

11 This invention will be better understood from the  
Experimental Details which follow. However, one skilled  
in the art will readily appreciate that the specific  
methods and results discussed are merely illustrative of  
the invention as described more fully in the claims which  
follow thereafter.

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## EXPERIMENTAL DETAILS

### First Series of Experiments

#### 5 Methods and Materials

Preparation of Red Blood Cell ghosts loaded with either indicator dye, peptide (antigen) or protein.

11 Lyse either human or sheep red blood cells in hypotonic KCl buffer containing 5 mM ATP and either a) indicator dye such as lucifer yellow at 5 mg/ml, b) peptides such as fluorescence-labelled ovalbumin, c) proteins, or d) lysate of tumor cells at 4°C for 20 mins. The same procedure can be done without adding ATP to the red blood cells.

17 Reseal ghosts in hypertonic KCl buffer to achieve isotonicity at 37°C for 40 mins.

Wash cells several times in phosphate buffered saline.

23 Opsonize (using published techniques) the red cells with the appropriate ligand which could be a) immunoglobulin (IgG), b) complement component C3b, c) complement component C3bi, d) maleic anhydride or others.

29 Add these opsonized ATP/peptide red blood cell resealed ghosts to isolated human dendritic cells obtained from either blood, bone marrow, brain, liver, skin or lymph nodes. Let the dendritic cells ingest the opsonized

ATP/peptide red blood cell resealed ghosts for several (3-24) hours with the appropriate cytokine such as GCSF, IL4, GMCSF, gamma interferon. The peptide will then be transferred from the phagolysosomes to other cytoplasmic compartments and eventually will be expressed as MHC class I antigens on the surface of the dendritic cells.

11 Either a) co-incubate these dendritic cells with lymphocytes in vitro for 6 hrs and then reinject the lymphocytes into the patient or b) inject these dendritic cells directly into the patient.

17 These dendritic cells should then induce class I MHC presentation and elicit cytotoxic T cells against the desired antigen. For example this method may be effective in generating an immunological response against tumors or microbial agents.

Opsonization of the red blood cell ghosts with C3bi enhances the transfer of lucifer yellow to the cytoplasm.

23 Shown herein is preparation of opsonized ATP/peptide red blood cell resealed ghosts containing either an indicator dye such as lucifer yellow (LY) or a peptide such as fluorescein-conjugated ovalbumin peptide (amino acids 257-264).

29 These opsonized ATP/peptide red blood cell resealed ghosts are now ingested by either mouse microglial cells (an antigen presenting macrophage like cell found in the brain) (Figure 1), human blood monocyte derived dendritic



Table 1: Mouse Bone marrow derived dendritic cells prepared as described in Figure 3 were incubated with the appropriate isogenic spleen derived CD8 lymphocytes for several hours and then the CTL response was assayed by radiolabeled thymidine incorporation into the lymphocytes indicating a proliferative response mediated via class I MHC.

Table 1. Class I antigen presentation via Ova peptide

Mouse bone derived dendritic cells treated with:	CTL Assay- <sup>3</sup> H-thymidine uptake (proliferation assay-cpm) *
Soluble Ova protein (10 mg/ml)	160,000
Soluble Ova peptide (100 ng/ml)	90,000
E(IgG) loaded with ATP and Ova peptide (100 ng/ml)	130,000
E(IgG) loaded with ATP and Ova peptide (10 ng/ml)	22,000
E(IgG) loaded with apyrase and Ova peptide (10 ng/ml)	23,000

\* in the absence of added antigen or with RBC's: cpms were <500.

Second Series of Experiments

To determine whether P2X<sub>7</sub> receptors mediate permeabilization of molecules from phago-lysosomes to the cytoplasmic matrix, prepared were IgG-coated resealed sheep red blood cell ghosts (IgG-rRBCg) containing the fluorescent dye Lucifer Yellow (LY) with or without ATP, and these ghosts were incubated with monolayers of J774 macrophage-like cells at 37°C. In IgG-rRBCg lacking ATP, apyrase was loaded into the RBCg to hydrolyze endogenous ATP. After 60 minutes, monolayers were briefly exposed to distilled water to lyse uningested IgG-rRBCg+LY+ATP or IgG-rRBCg+LY and examined by phase and fluorescence microscopy. As expected, >90% of the macrophages ingested one or more IgG-rRBCg+LY+ATP or IgG-rRBCg+LY. These brightly fluorescent IgG-rRBCgs were contained in phago-lysosomes in the J774 cells' cytoplasm. About 50% of the J774 cells that had ingested IgG-rRBCg+LY+ATP contained LY in their cytoplasmic and nuclear matrices (see Figure 4A). The LY remained in the cytoplasmic and nuclear matrices of J774 cells that ingested IgGrRBCg+LY+ATP for 48 hours, the longest time point examined. In contrast, no LY was detected in the cytoplasmic or nuclear matrices of J774 cells that had ingested IgGrRBCg+LY at any time from 0.5 to 48 hours (Figure 4B). Similar results were obtained using thioglycollate elicited mouse peritoneal macrophages (data not shown). To confirm these results J774 cells which lack P2X<sub>7</sub> receptors (J774-P2X<sub>7</sub> null) were used. These cells were selected and characterized as described (7). About 60% of J774-P2X<sub>7</sub> null cells ingested



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washed to remove free peptide, and incubated at 37°C for 1 hour with monolayers of macrophages or of J774 cells. (Ghosts containing TPHPARIGL, LY and ATP will be used in the initial experiments to confirm that these ghosts are being ingested and that ATP in them activates P2X<sub>7</sub> receptors that allow LY into the cytoplasmic and nuclear matrices. Furthermore, as described above endogenous ATP from IgGrRBCg+TPHPARIGL+LY will be hydrolyzed by loading these ghosts with apyrase.) Uningested IgGrRBCg will be removed by lysis as described above, the peritoneal macrophages or J774 cells will be further incubated at 37°C for varying time periods to allow processing of the TPHPARIGL. The macrophages or J774 cells then will be labeled with <sup>51</sup>Cr and incubated with various ratios of CTL0805B cells (e.g., 10-50 CTL0805B cells per target cell) for 4 hours at 37°C, at which time the medium from these cultures will be collected, sedimented to remove detached but unlysed cells, and assayed for <sup>51</sup>Cr release as a measure of cytotoxicity, as described (8). Positive controls should show that CTL0805B will lyse macrophages, wild type J774 cells, or J774-P2X<sub>7</sub> null cells that were pre-loaded incubated with high concentrations of TPHPARIGL peptide prior to incubating them with CTL0805B. CTL0805B should not lyse the following cells: 1. Macrophages or J774 cells treated with cytochalasin D to prevent ingestion of the IgGrRBCg+TPHPARIGL+ATP or of the IgGrRBCg+TPHPARIGL. 2. J774-P2X<sub>7</sub> null cells incubated with IgGrRBCg+TPHPARIGL+ATP or IgGrRBCg+TPHPARIGL. 3. Macrophages or J774 cells treated with Brefeldin A to prevent transport of TPHPARGL-loaded Class I MHC proteins from the endoplasmic reticulum to the surface. 4.





containing TPHPARIGL with or without ATP. Uningested IgGrRBCg+TPHPARIGL will be lysed, and these APCs will be administered to Balb/c mice weekly for 3-6 weeks intraperitoneally or subcutaneously. The mice then will be sacrificed and their spleen and regional lymph node cells tested for CTL activity against TPHPARIGL-pulsed J774 cells (as described in 8), and for helper T-lymphocytes using <sup>3</sup>H-thymidine incorporation or IL-2 production using X-irradiated lac-Z transfected Balb/c 3T3 cells as stimulators (9).

Anticipated results: rRBCg+TPHPARIGL+ATP, IgGrRBCg TPHPARIGEL+ATP, or macrophages or dendritic cells that ingested IgGrRBCg TPHPARIGEL+ATP will induce formation of CTLs while rRBCg+TPHPARIGEL, IgGrRBCg+TPHPARIGEL, or macrophages or dendritic cells that ingested IgGrRBCg+TPHPARIGEL will not. In contrast, all preparation will induce activation of helper T-lymphocytes.

Next, after obtaining positive results in the experiments described in 2 above, P2X<sub>7</sub>-knock out mice will be obtained and it will be determined whether they are incapable of mounting a helper or CTL response to IgGrRBCg+TPHPARIGL+ATP.

The mechanism by which APCs activate helper and cytotoxic lymphocytes to react to peptide antigens, including peptides with altered amino acid sequences from mutated tumor-cell proteins, is central to current immunotherapeutic approaches to cancer. The experiments

herein will provide insight into the cellular mechanism by which apoptotic cells deliver antigenic peptides to Class I MHC of APCs and may uncover a novel and potentially clinically useful protocol for activating cytotoxic and helper T-lymphocytes.

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